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Thesis

THE ANTIGENIC PROPERTIES OF SYNTHETIC GELATIN COMPOUNDS

by

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(S.B., Massachusetts Institute of Technology, 1933)

submitted in partial fulfilment of the

requirements for the degree of

Master of Arts

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I. Review of the literature.

A. The non-antigenicity of gelatin.

Most proteins are antigenic. Gelatin has repeatedly been shown to be non-antigenic.

One of the first observations made to show that gelatin is non-antigenic was by Wells.[24] He tried anaphylaxis experiments on guinea-pigs and got negative reactions. He beleived that gelatin was non antigenic because it lacked aromatic amino acids and would not therefore produce toxic portions according to Vaughan's Theory of Anaphylaxis.

Pabes and Regazzi[17] sought to induce anaphylactic reactions in rabbits by repeatedly injecting both normal and hydrolysed gelatin and obtained negative results.

Kahn and McNeil[8] attempted to get complememt fixation reactions by injecting gelatin. They injected rabbits intravenously at twenty-four hour intervals with successive doses of 50, 75 and 100 milligrams of gelatin and bled eight days after the last injection. They obtained negative results. This is not surprising in view of the fact that their doses were large and only a few were given.

The most systematic and exhaustive work ever done to show the non antigenicity of gelatin was reported by Starin.[21]

For the preparation of his antigens, Starin purified a sample of the French "Gold Leaf" gelatin by repeated precipitation in alcohol and tested the resulting product for protein impurities. Since this method may be of some interest I quote from Starin:

"Gelatin can be separated and quantitatively determined by adding to the gelatin solution a mixture of one part of picric acid (sat. aq. sol.) plus four parts of absolute alcohol. At 80°C all other proteins are precitated while the gelatin is held in solution. On evaporation of the alcohol following filtration, the gelatin is precipitated quantitatively, as cooling progresses. The application of this method to the product obtained, gave no indication of the presence of proteins other than gelatin."

Using the protein purified as above, Starin carried out anaphylaxis, complement fixation, precipitin and meiostagmin reactions. He also tried to demonstrate the production of proteolytic ferments.

For the anaphylaxis experiments, he used rabbits, guinea-pigs and dogs. The animals were observed for the following symptoms: (a) general (b) variations in temperature following a second injection (c) character of the blood pressure (in dogs) (d) changes in the coagulation time of the blood. (e) necropsy findings.

The rabbits were sensitized with intraperitoneal doses of gelatin ranging from 0.04-0.1 of a gram. The second and third injections were 5-10 times the sensitizing doses and were given both intravenously and intraabdominally allowing 10, 12, and 16 days to intervene between injections. Only doubtful and negative symptoms were observed.

Starin also makes the interesting observation that the intravenous injection of more than 0.5 gram of gelatin is

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fatal to rabbits.

The anaphylaxis experiments with guinea pigs were carried out in the same general way except that smaller doses were given (0.005-.05 grams). The routes of injection were intra-abdominally, intracardial and intrajugular. Only doubtful and negative symptoms were observed.

Two young dogs were also injected intraabdominally with initial doses of 0.1 to 0.5 gram of gelatin followed by doses of 2 and 3 grams through the femoral vein. No significent changes were observed in the blood clotting time of the dogs, nor did any necropsy findings indicate anaphylaxis.

Starin also tried to produce precipitins against gelatin using both the intraabdominal and intravenous routes singly and together. The total amount of gelatin injected varied from 0.6 to 12 grams. The period of injection varied from a few days to 9 months and the number of doses from 3 to 25. Precipitin and complement fixation tests were consistently negative.

Since the meiostagmin reaction and the production of proteolytic ferments have fallen into some disrepute as criteria of antigenicity they will not be discussed here.

Arthus[2], however, reported that he was able to sensitize rabbits with gelatin by intravenous injection. After five or six injections, he observed lack of absorption of the injected material and abscess formation, at the point of injection, such as is found in local anaphylaxis or the Arthus phenomenon.

Bruynoghe and Vassiliadis[4] found positive complement fixation and negative precipatation using gelatin as an antigin. They,

fatal to rabbits.

The anaphylaxis experiments with rabbits were carried out in the same manner as except intramuscular doses were given 0.005-.05 grams. The reaction of injection were indurations, intracutaneous and intratunular. Only doubtful and negative symptoms were observed.

Two young dogs were also injected intravenously with initial doses of 0.1 to 0.5 gram of gelatin followed by doses of 2 and 4 grams through the femoral vein. No significant changes were observed in the blood clotting time of the dogs, nor did any anaphylactic tendency indicate anaphylaxis.

Gelatin also tried to produce immunization against gelatin using both the intravenous and intraperitoneal routes single and together. The total amount of gelatin injected varied from 0.5 to 15 grams. The period of injection varied from 2 to days to 3 months and the number of doses from 2 to. Treatment and some weekly injection tests are uniformly negative.

Since the main reaction and the production of protein in tissues have fallen into some disrepute as criteria of anaphylaxis, they might be disregarded here.

Rabbits, however, reported that he was able to sensitize rabbits with egg albumin by intravenous injection. After five or six injections, he observed lack of sensitization of the injected material and absence formation, at the point of injection. Death of the animal in four anaphylaxis or the stance skin reaction.

Strongyne and Vosselliers found positive skin reaction and negative precipitation tests with as antigen. Ker,

however, used a very large antigenic "dose", one half of the largest quantity of gelatin which when coupled to anilin will allow complete hemolysis in a hemolytic system.

Using antisera to gelatin and anilin-gelatin they did not get fixation with anilin-gelatin with either antiserum but gave fixation with gelatin alone. These results are very peculiar and due to the lack of specific details cannot be evaluated properly.

Despite a few discrepancies in the literature, the consensus of opinion is that gelatin is non-antigenic. The very careful work of Starin is especially convincing. It must be remembered, however, that the gelatin used in these experiments was not all from the same source. All gelatins are not of the same composition. They will vary with the type of collagen used in its prepartion, and the method of hydrolysis, among other factors. Gelatin is not a single chemical entity as has been demonstrated by Krishmanurti and Svedberg.[9] From an ultracentrifugal study of gelatin solutions, they find that gelatin solutions contain a mixture of proteins varying in molecular weight from 11,000 to 70,000.

Furthermore, not all investigators have taken precautions to separate foreign proteins from the gelatin. These considerations might explain some of the discrepancies in the literature.

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Furthermore, not all investigators have taken precautions to separate foreign proteins from the gelatin. These considerations might explain some of the discrepancies in the literature.

B. Compounds related to gelatin.

In connection with the non-antigenicity of gelatin it is interesting to note that glue, a substance related to gelatin is non antigenic, as has been demonstrated by Ramsdell and Walzer.[18] Since glue is a potent atopen in human supersensitiveness, they investigated its antigenic effect in the guinea-pig and rabbit. Using the Dale method they obtained only weak and submaximal contractions in the uterine strips from injected animals. They conclude therefore that glue is non antigenic.

Loiselleur and Urbain[14] found collagen to be antigenic. They prepare collagen according to the directions of Nageatte. This method has the advantage that no heat is required to dissolve the collagen thereby eliminating one cause of denaturation. They prepare the collagen as follows:

The tendons of a rat's tail were suspended in a very dilute solution of acetic acid and allowed to swell; the collagen dissolved in the acid was separated from the cell residue by centrifuging and preserved on ice in the presence of ether. The solution had a pH of 3.89 and its protein content was 1.93 grams per liter.

Two rabbits received 6 subcutaneous injections of collagen from 1-6 cc. After the fifth injection the edema characteristic of an Arthus phenomenon appeared. At this point the complement fixation reaction became positive. The fixation tests were controlled by using rat serum as an antigen. No fixation was obtained with this control. The authors therefore conclude that the reaction is specific for collagen.

4. Compounds related to gelatin.

In connection with the non-antigenicity of gelatin it is interesting to note that glue, a substance related to gelatin is non antigenic, as has been demonstrated by Samsoff and Bulger. Since glue is a potent stimulus in human experimentation, they investigated its antigenic effect in the guinea-pig and rabbit. Using the Isle method they obtained only weak and subcutaneous contractions in the guinea-pigs from injected animals. They conclude therefore that glue is non antigenic.

Coleslaw and Brodin found collagen to be antigenic. They prepare collagen according to the directions of Langstein. This method has the advantage that no heat is required to dissolve the collagen thereby eliminating one cause of denaturation. They prepare the collagen as follows:

The tendons of a rat's tail were suspended in a very dilute solution of acetic acid and allowed to swell; the collagen dissolved in the acid was separated from the tail residue by centrifuging and precipitated on the addition of ether. The solution had a pH of 5. and its protein content was 1.52 grams per liter.

Two rabbits received 6 cmcc tenths injection of collagen from 1-6 cc. After the first injection the shock phenomenon appeared. At this point the development of an anaphylaxis became positive. The first on testing was controlled by using rat serum as an antigen. The titration was obtained with this antiserum. The results therefore indicate that the reaction is specific for collagen.

They also tested the action of radium on this antigen and found that irradiation decreased the viscosity and also reduced the antibody titre to one-third of the original; the same effects are obtained by heating the antigen at 100° C.

These results are at present unconfirmed. If confirmed, it would cause a fundamental revision of our ideas concerning the non-antigenicity of gelatin. This point will be discussed later.

C. Synthetic gelatin compounds as antigens.

Although gelatin itself is non-antigenic there are some reports in the literature in which gelatin has been made antigenic by coupling with aromatic groups.

The theoretical background for this work is discussed by Wells.[23b] From the many analyses of gelatin that have been made, it has been found that gelatin is characterised by a lack of aromatic radicals. It contains no tryptophan or tyrosin and only a very small amount of phenylalanine. Since most antigenic proteins contain these amino-acids, it has been thought that the non-antigenicity of gelatin is due to a lack of these amino acids. In support of this contention are the following facts:

(1) It has been shown by Vaughan[23b] that in the toxic and non-toxic portions formed in the cleavage of proteins, the toxic fractions contain aromatic radicals. Gelatin yields no toxic fraction because it is deficient in aromatic groups.

(2) Proteins deficient in amino acids other than aromatic radicals are antigenic. For instance, zein lacks tryptophan, lysin and glycine, but has a large proportion of tyrosin. Gliadin lacks glycine, while casein as deficient in cystin and glycine. Still, all these proteins are strongly antigenic.

(3) The protamins are non antigenic but they consist mostly of diamino acids and a small quantity of a few of the monoamino acids. Lysin is not present in the protein, hordein as well as arginin and histidin. This indicates that the diamino acids are not of importance in determining antigenicity.

4. Synthetic gelatin properties and uses.

Although gelatin itself is non-antigenic there are some reports in the literature in which gelatin has been made antigenic by coupling with aromatic groups.

The theoretical background for this work is discussed by Haas. From the many analyses of gelatin that have been made, it has been found that gelatin is characterized by a lack of aromatic radicals. It contains no tryptophan or tyrosin and only a very small amount of cyclic imino-acids. Since most of the amino groups contain these amino-acids, it has been thought that the non-antigenicity of gelatin is due to a lack of these amino acids. In support of this conclusion are the following facts:

(1) It has been shown by Vaughan that in the tryptic and non-tryptic portions formed in the digestion of proteins, the basic fractions contain aromatic radicals. Gelatin yields no basic tryptic products. It is still lacking in aromatic groups.

(2) Crystals sufficient in amino acids other than aromatic radicals are antigenic. For instance, egg albumin tryptic digest and glycine, all has a large percentage of tyrosin. Glycine glycine, which contains no sufficient in glycin and glycine, still, all these crystals are strongly antigenic.

(3) The proteins are non antigenic but their constituent moiety of amino acids and a small density or a lack of the mono-amino acids. Lysin is not present in the proteins, protein are antigenic and gelatin. This indicates that the amino acids are not of importance in antigenicity and activity.

(4) It has been shown by Obermeyer and Pick[23b] that the aromatic radicals of proteins are important in determining specificity. This is effected by introducing into the protein molecule substances which are known to react with the benzene rings. Proteins thus altered lose their species specificity entirely.

If aromatic radicals are necessary for antigenicity, it follows that their introduction into the protein molecule might make gelatin antigenic. This has been attempted by a few investigators.

Adant[1] claims to have produced precipitins to gelatin-diazoanilin. Using anti-gelatin-diazo-anilin serum, he obtained precipitins with both the diazo compound and gelatin, although the latter was not antigenic. He also obtained specific inhibition of precipitation with other anilin proteins.

Bruynoghe and Vassiliadis[4] observed that anti-gelatin-diazo-anilin fixed complement in the presence of gelatin but not with the homologous antigen.

Hooker and Boyd[5] found that anti-gelatin-diazo-arsanilic acid gave precipitin reactions with both casein-diazo-arsanilic acid and ovalbumin diazo-arsanilic acid but not with the homologous antigen. Anti-gelatin-diazo-arsanilic acid also fixed alexin in the presence of casein and egg-white-arsanilic acids, The antiserum was specificly inhibited by both phenol-diazo-arsanilic acid as well as by the homologous compound.

They reach the conclusion that "antigenicity may be conferred upon gelatin by coupling it with an unsaturated cyclic

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They reach the conclusion that "antigenicity may be conferred upon substrate by coupling it with a chemically active

compound" but add that the results, "do not show that non antigenicity of gelatin is due solely to its deficiency in aromatic amino-acids." Other investigators using different prosthethic aromatic groups were not able to confer antigenicity on gelatin but obtained only combining effects similar to haptens.

Landsteiner[10] found that diazotised metanilic acid coupled with gelatin gave precipitins with anti-horse-serum coupled with m-diazo-henzenesuphonic acid. This gelatin compound specificly inhibited reactions between the diazo proteins and its antiserum using gelatin diazo-arsanilic acid. He obtained the same results with the homologous antihorse serum compound.

Medveczky and Uhrovits[15] found that henzoylated gelatin did not give reactions with its antiserum, but give very strong reactions with antisera for henzoylated horse serum and benzlylated typhoid bacilli. The antiserum for benzoylated bacilli reacted with a solution of gelatin containing 5 per cent of the henzoyl radical in a titer of 1: 10,000,000.

Benzoylated gelatin was strongly anaphylactic to guinea pigs sensitized with the benzoylated typhoid bacilli, so that a dose of 0.0000 15 grams is fatal.

These authors also note that henzoylated gelatin has a much more powerful reactivity than the homologous proteins in their relative precipitating power. Hopkins and Wormall[7b] also observed this effect in their phenylureidogelatin compounds. They found that in the precipitin and complement fixation reactions with phenyluredogelatin and the homologous serum protein compounds, that the zone of maximal precipitation or complete

complement fixation is present in much more dilute solutions of the gelatin compounds. They note that the difference in activity is not dependent on the number of phenylureido groups coupled with the protein. No explanation is given for this phenomenon.

Hopkins and Normall[7a] introduced an entirely new method for coupling additional groups to the protein molecule. The older methods altered the structure of the protein by acids, alkalies, enzymes, nitration, iodization, etc. Landsteiner was the first to use azoproteins, produced by the reaction of various diazonium salts with proteins in alkaline solution. This is the method usually employed for introducing groups into the protein molecule. Coupling is supposed to take place only with the histidin and tyrosin in the protein.

The phenlisocyanate proteins can be prepared by coupling isocyanates to the proteins in a medium of pH 8. The reaction involves the free amino groups. Analysis of these proteins shows that the increase in the isocyanate content is accompanied by a corresponding decrease in the free amino nitrogen as found by bromin determination on p-bromophenylureido-proteins. The reaction may be represented as follows:

$$R-\underset{\underset{NH_2}{|}}{CH}- + OC{:}N\cdot C_6H_5 \longrightarrow R-\underset{\underset{NH\cdot CO\cdot NH\cdot C_6H_5}{|}}{CH}-$$

This method of introducing additional groups into the protein molecule has several advantages over the other methods used. The linkage closely resembles the peptid linkage, the

complement fixation is present in much more dilute solutions of the gelatin components. They prove that the difference in activity is not dependent on the number of phenylcarbo groups reacted with the protein. The explanation is given for this phenomenon.

Coupling was formerly introduced on antigenic properties for coupling additional groups to the protein molecule. The other methods altered the structure of the protein by acid, alkaline, oxygen, nitrite, iodination, etc. Landsteiner has the advantage to use azodyestuffs, produced by the reaction of various diazonium salts with proteins in alkaline solution. This is the method usually employed for hydrophilic groups into the protein molecule. Coupling is supposed to take place only with the histidine and tyrosin in the protein.

The benzidoyl-amino protein can be prepared by coupling isocyanate to the protein in a medium of CH_3. The reaction involves the free amino groups. Analysis of their reactions show that the increase in the isocyanate content is accompanied by a corresponding decrease in the free amino nitrogen. It was found to begin introduction on o-tricosanylurea-protein. The reaction may be represented as follows:

$$R-CH(NH_2) + OCN\,C_6H_5 \rightarrow R-CH(NH)\,CO\,NH\,C_6H_5$$

This method of introducing additional groups into the protein molecule has several advantages over the other methods. The linkage closely resembles the peptid linkage, the

excess of isocyanate is rapidly destroyed, and its products are easily removed from the protein solution.

These authors obtained only combining effects with their phenylureidogelatin compounds. They got marked precipitin and complement fixation reactions with antisera to phenylureido-horse-serum-globulin. They also noted that precipitin reactions with the gelatin compounds are best carried out at 15-20°C or lower temperatures, since there is a tendency for the pri-cipitates to dissolve at 37°. Despite injection over a long period, they were unable to obtain procipitin or fixation reactions with phenylureido- and p-bromophenylureido gelatin using the homologous or heterologous phenylureido-proteins.

They conclude therefore that these gelatin compounds are non-antigenic and that the non-antigenicity of gelatin is not due solely to a deficiency in aromatic groups.

C. Discussion.

There is a peculiar lack of uniformity in the antigenic reactions of the synthethic gelatin compounds as reported by different investigators. In the first place Adant's[1] results are very hard to explain. Using an antidiazo-anilin-gelatin he observed precipitation with both gelatin and the homologous antigen although his gelatin was non-antigenic. This evidences that two antibodies were produced by gelatin-diazoanilin. It indicates that once having made gelatin antigenic by additional groups, gelatin itself will act as a precipitable hapten. Hooker and Boyd[6] were unable to get either precipitin or inhibition reactions with antidiazoarsanilic acid gelatin using gelatin itself.

A further complication is introduced when we consider the work of Bruynoghe and Vassiliadis.[4] They obtain fixation with gelatin and not with the homologous antigen using antianilin gelatin. According to our present knowledge we would at least expect fixation with the homologous antigen. In view of these controversial findings the antigenicity of gelatin-diazo-anilin seems to be very doubtful. The author has therefore thought that a repetition of this work would yield interesting information.

According to the findings of Landsteiner;[10] Medveczky and Uhrovits[15] and Hopkins and Wormall,[7b] there is no doubt that substituted gelatins are effective precipitable haptens. Hopkins and Wormall[7b] reach the conclusion that to make gelatin antigenic

C. Discussion.

There is a peculiar lack of uniformity in the antigenic reactions of the synthetic gelatin compounds as reported by different investigators. In the first place Abderhalden's results are very hard to explain. Using an antihuman-serum-gelatin he observed precipitation with both gelatin and the homologous antigen although his gelatin was non-antigenic. This evidence that the antibodies were produced by gelatin-fragments. It implies that only having many gelatin antigens by artificial groups, gelatin itself will act as a precipitinogen antigen. Doerr and Sonn are unable to get either precipitin or hemagglutination results due with antihaptenresults with gelatin being positive instead.

A further complication is introduced when we consider the work of Pungche on "gelatinin". They obtain fixation with gelatin and not with the antiserum antigen using antihuman gelatin. According to our present knowledge we would at least expect fixation with the homologous antigen. In view of these controversial findings the antigenicity of gelatin-antihaptens seems to be very doubtful. The author has attempted somewhat the repetition of this work using gelatin-antihaptens for comparison.

Prior to the judging of immunization, Morgenroth and Sherrick are inclined and correct, there is no doubt that antibodies are produced from gelatin antigens. Doerr and Sonn have shown the immunization that to make gelatin a specific

in the sense of engendering specific antibodies, the introduction of aromatic groups is not sufficient.

The evidence for the antigenicity of gelatin diazoarsanilic acid is not entirely satisfactory. Although positive fixation and precipitin reactions were obtained, these did not occur with the homologous but only with heterologous antigens.

E. Some aspects of the chemistry of gelatin and collagen.

In this section an attempt has been made to summarize briefly some of the recent literature relating to the structure and chemical properties of gelatin and collagen. One of the very voluminous literature on the subject, the author attempted to bring out only those points that might have immunological significance.

Collagen is one of the most important of the proteins in the several connective tissues of the body. The organic material of the bones, tendons, cartilage and skin is largely composed of collagen.

Ssadikoff[19] has made a study of the structure of collagen and comes to the conclusion that the collagen micelle contains only glycin, alanin, prolin, leucin, arginin, lysin, asparagin, and glutamic acid, all other amino acids that might be found on analysis are impurities. He bases his conclusions on the result of x ray analysis, enzymatic action and the hydrolytic products of gelatin as determined by Dakin.[32]

Through study of the hydrolysis products of gelatin Dakin found the following amino acids:

glycin	25.5%	prolin	14.1%
alanin	8.7%	leucin	7.1%
arginin	8.2%	asparagin	3.4%
lysin	5.9%	glutamicacid	5.8%

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Collagen is one of the most important of the proteins in the several connective tissues of the body. The organic material of the bones, tendons, cartilage and skin is largely composed of collagen.

Sandiford[19] has made a study of the structure of collagen and comes to the conclusion that the collagen micelle contains only glycin, alanin, prolin, leucin, arginin, lysin, asparagin, and glutamic acid. All other amino acids that will be found on analysis are impurities. He bases his conclusions on the result of x ray analysis, organic acetin and the hydrolytic products of gelatin as determined by Smith.[34]

Through study of the hydrolytic products of gelatin Smith found the following amino acids:

glycin	25.5%	prolin	14.1%
alanin	8.7%	leucin	7.1%
arginin	9.3%	asparagin	5.4%
lysin	5.9%	glutamic acid	6.0%

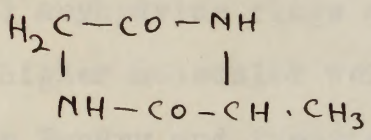
serin	0.04%	valin	0%
phenylalanin	1.4%	cystin	0%
hystidine	0.9%	tyrosin	0.01%
		tryptophane	0%

Ssadikoff believes that in collagen the last group of amino acids as absent since they are present in very small percentage.

Furthurmore x ray analysis seem to bear out this point.[5] The molecular unit as found by this method for collagen is 685. This indicates that the micelle of collagen is built up of four cyclopeptide linkages as follows:

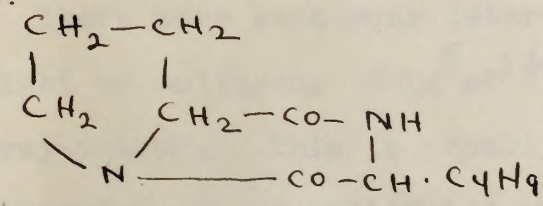
Two molecules of cycloglycalanin	114+114 =	228A
One molecule of cycloprolyl-leucin	=	198B
One molecule of cyclo asparagyl-arginin (or lysin)	=	266C
		692 Total

A.



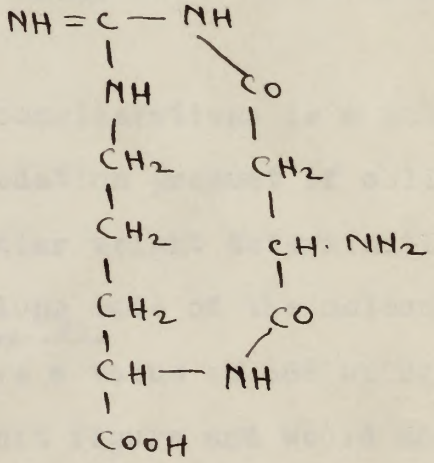
cyclo-glycylalanin

B.



cyclo-prolyl-leucin

C



cyclo-asparagylarginin

serin 0.04% valin 0%

phenylalanin 1.4% cystin 0%

hystidine 0.9% tyrosin 0.01%

tryptophane 0%

Saadhoff believes that in collagen the last group of amino acids as absent since they are present in very small percentage.

Furthermore X ray analysis seem to bear out this point.

The molecular unit as found by this method for collagen is 555.

This indicates that the micelle of collagen is built up of four cyclopeptide linkages as follows:

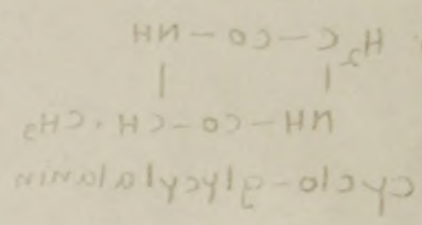
Two molecules of cyclogly-leucin 114+114 = 228A

One molecule of cyclopro-ly-leucin = 1083

One molecule of cyclo asparagy-arginin = 2160

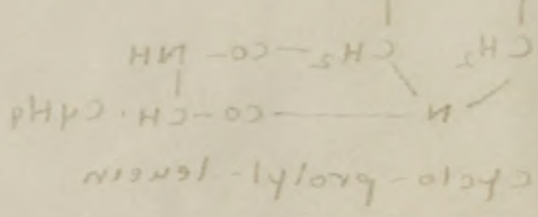
(or lysin) 692 Total

A



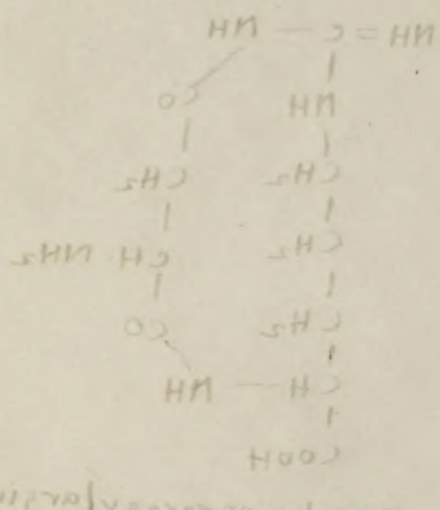
cyclo-glycylalanin

B



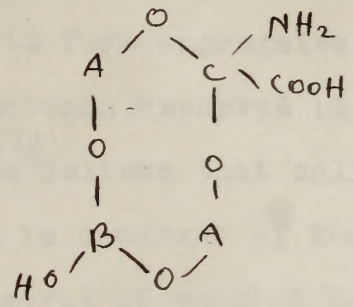
cyclo-prolyl-leucin

C



cyclo-asparagylarginin

The formula for the collagen micelle would therefore be as follows:



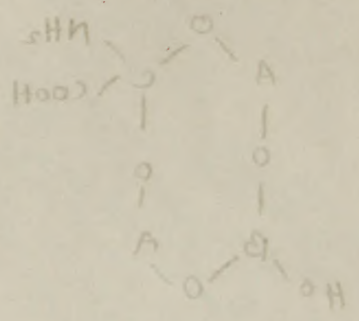
Adjacent polypeptide rings are bound together by means of the enolic hydroxyl linkage. The free group (OH, NH, COOH) enable one unit to combine with another.

Collagen may therefore be considered as a cyclopeptide combined in an enolic anyhydride ring. In the hydrolysis of collagen to gelatin a hydrolysis occurs with a breaking of the enol anyhydride rings and the subsequent formation of aggregates of higher molecular weight. These views are also brought out by Cohn, Hendry, and Prentiss.[5]

Gelatin according to the above considerations is a polymerised rather than hydrolytic digredation product of collagen. This seems to be borne out by molecular weight determinations.

There have been many determinations made of the molecular weight of collagen. Cohn[5] and his associates all give a value of 685 using the x ray method. This is proably a unit figure and would most likely have to be multiplied by some whole number to give the correct molecular weight. Herzog[3a] found the molecular weight vary between 2000 and 2600 by determining the diffusion

The formula for the collagen acetate would therefore be as follows:



Adjacent polypeptide rings are bound together by means of the amolin hydroxyl linkage. The free group (OH, NH, COOH) enable the unit to combine with another.

Collagen may therefore be considered as a polypeptide combined in an amolin anhydride ring. In the hydrolysis of collagen to gelatin a hydrolysis occurs with a breaking of the amol anhydride rings and the subsequent formation of aggregates of higher molecular weight. These **views** are also brought out by Cohn Hendry and Prentiss.

Gelatin according to the above considerations is a polymerised rather than hydrolytic degradation product of collagen. This seems to be borne out by molecular weight determinations.

There have been many determinations made of the molecular weight of collagen. Cohn et al give a value of 888 using the x ray method. This is probably a unit figure and would most likely have to be multiplied by some whole number to give the correct molecular weight. Harris found the molecular weight very between 2000 and 2800 by determining the diffusion

coefficient of a polydispersed solution of collagen in m-cresol. This value may be accepted as a maximal molecular weight since proteins tend to form aggregates in solution. There are many other determinations reported in the literature.

Herzog and Janke[3d] believe that collagen is not a single chemical entity but is composed of two substances.

The molecular weight of gelatin has been determined by Svedberg[9] using the ultracentrafuge. He found that gelatin is a mixture of proteins varying in molecular weight from 11,000 to 70,000. He considers values below 11,000 as degradation products His evidence seems to indicate that the true molecular weight of gelatin is 11,000 and that the higher values are merely molecular aggregations.

If we accept 2600 as the molecular weight of collagen and 11,000 as the molecular weight of gelatin, then in the change from collagen to gelatin a polymerization occurs instead of a hydrolytic breakdown as ordinarily assumed.

The action of enzymes on collagen[20] furnish very important clues to its structure. Trypsin free from collagenase does not attack collagen. This is a characteristic of the action of trypsin on cyclic structures. It might also be noted at this point that ninhydrin, a reagent which reacts with proteins, polypeptids and alpha amino acids does not give a blue color with collagen. Pepsin however acts on collagen in acid medium to give non gelling peptised products. Collagenase free from trypsin peptises collagen in alkaline solution without forming

amino acids.

Collagens from different tissues do not have the same composition.[3b] It might be possible that collagen exhibits a tissue specificity. There seem to be chemical differences in the bone collagen of male and female animals.[3c]

F. Theoretical considerations

No satisfactory explanation has been advanced to date to explain the non-antigenicity of gelatin. In order to look at the problem intelligently we may well ask, to what factors in general do proteins owe their antigenicity? That knowledge we do have on the subject is very vague.

The first problem is the matter of definition. For as Wells[23a] notes in his review "An antigen is any substance which introduced into the tissues or circulating fluids of an animal causes the appearance in these fluids sooner or later of substances which specificaly react with the antigenic substance i.e. specific antibodies."

It is quite possible that antibodies may be produced within the cells and remain fixed and therefore not appear in the blood.

In order to exhibit antigenicity, the substance injected must be foriegn to the tissues of the body so that appropiate defence mechanisms can be set up resulting in the formation of antibodies. As far as we know, gelatin is certainly foriegn to the animal body. Collagen is not foriegn to the animal body. However collagens from different animals have been shown to differ in chemical composition[3b] and might therefore be expected to stimulate the production of antibodies if injected into another animal.

Also antigenicity seems to be dependent on the size of the molecule. Gelatin contains varying proteins in molecular

weight from about 11,000 to 70,000, which is of the order of magnitude of other antigenic proteins. Molecular aggregations do not seem necessary since in the concentrations used for immunizing, many proteins, egg albumin, for example, are monodispersed.

If the work on collagen can be confirmed, we may have to discard the idea that aromatic rings are necessary for antigenicity. It is possible that no particular amino acids are necessary for the antigenicity of proteins. There are claims in the literature that some complex carbohydrates are antigenic. In carbohydrates the building stones are usually simple monosaccharides. In any one carbohydrate the structural units do not have the diversity of the amino acids. Reasoning thus by analogy, it does not seem necessary to postulate the existance of certain amino acids in a protein in order to make it antigenic.

From purely chemical considerations it is difficult to explain the non-antigenicity of gelatin. During the change of collagen to gelatin no part of the molecule seems to be split away, the molecule is polymerised in fact. The question might then be asked, why should collagen be antigenic and gelatin not. Gelatin possibly lacks the proper internal arrangement of the amino acids. We know that under the influence of heat egg albumin is denatured and loses its antigenicity.[23c] Casein however does not loose its antigenic properties on heating. The "antigenic pattern" of some proteins is more stable than others. It might be possible to make gelatin antigenic by meerly altering

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the configuration of the molecule by some physical means. It was thought that a plastein synthesis of a gelatin digest might give a possible antigenic rearrangement of the molecule. Wastenys and Borsook[22] however could not make a plastein from gelatin. It is also rather interesting that glue is a powerful atopen in human supersentivness.

It was thought that gelatin might be non antigenic due to racemization. Gelatin has a very high specific rotation[38] ranging from-496.8 to -181.1 with wave lengths of light from 4339 to 6660 Eggalbumin[38] varies under some conditions from -83,9 to -27.5.

As a concluding thought, let us assume for the moment that by appropriate chemical procedures we could make gelatin fully antigenic. The results obtained would not necessarily be capable of general extension. Specificity and antigenicity are two concurrent factors. An antigenic substance is always specific. If specificity factors are absent then we cannot tell whether or not a substance is antigenic. We know that specificity varies with different proteins, and that it is possible to alter it by introducing additional groups. So that by solving the problem for gelatin we might only get results applicable only to that protein. To carry this reasoning further, the structural changes produced by the chemical reactions employed might make gelatin antigenic so that the introduction of additional groups might meerly by an artifact. The additional group would then serve to change the specificty of an already antigenic molecule.

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Karsnys and Roesch[22] however could not make a gliadin from gelatin. It is also rather interesting that glue is a powerful stopper in human supersensitiveness.

It was thought that perhaps a might be non antigenic due to racemization. Gelatin has a very high specific rotation ranging from -429.6 to -1021.1 with wave lengths of light from 4359 to 6563 Angstrom[34] units under some conditions from -88.3 to -427.8.

As a concluding thought, let us assume for the moment that by appropriate chemical procedures we could make gelatin fully antigenic. The results obtained would not necessarily be capable of general extension. Specificity and antigenicity are two nonsurrent factors. An antigenic substance is always specific. If specificity factors are absent, we cannot tell whether or not a substance is antigenic. We know that specificity varies with different proteins, and that it is possible to alter it by introducing additional groups. So that by solving the problem for gelatin we might not get results applicable only to that protein. To carry this reasoning further, the structural changes produced by the chemical reactions employed might make gelatin antigenic so that the introduction of additional groups might merely by an artifact. The additional group would then serve to change the specificity of an already antigenic molecule.

II. Experimental work.

A. Preparation of nitro-proteins.

Landsteiner and Barron[11] have demonstrated that if a racemised protein is nitrated its antigenic properties would be restored. Gelatin, from its method of preparation, may be considered a racemised protein. It was therefore thought that nitration might make gelatin antigenic.

After trying several modifications of Lieben's[13] original procedure we prepared a nitro-gelatin in the following manner:

(1) Preparation of nitro-gelatin.

Twenty grams of gelatin (lot no 18613 Bacto Difco Brand controlled for non-antigenicity by Hooker and Boyd[6]) was sifted into 100 cc. of concentrated nitric acid. Nitration was allowed to continue for ten minutes. The temperature during nitration varied from 64°-70° C. The solution was immediately diluted to about 500 cc. with cold water. Solid ammonium sulphate was then added until precipitate was light yellow in color and resembled a sticky gum. It was suspended in 500 cc. of water and dissolved by adding alkali to a slight basic reaction of litmus. The resulting orange- colored solution was dialysed over night, subsequently sterilized by Berkefeld filtration and made isotonic with salt. The nitrogen content was found to be 3.68 mgs. of nitrogen per cc.

(2) Preparation of nitro-casein.

Twenty grams of casein (Pfandsteil, lot 6016) were sifted into 500 cc. of 20 per cent nitric acid and cooled under the tap during nitration. Small clumps were formed which did not

dissolve. The solution was colored a deep yellow. The clumps dissolved however, on the addition of alkali and the solution turned orange in color. The protein was precipitated by the addition of a minimal quantity of acid and then suspended in 250 cc. of water and dissolved by the addition of a minimal quantity of alkali. Since these solutions were only to be used as t as test antigens they were not sterilized.

(3) The preparation of nitro-albumin.

Twenty grams of albumin were sifted into 500 cc. of 1:3 nitric acid and cooled under the tap during nitration. The nitration formed an insoluble golden yellow precipitate of nitro-albumin which was filtered by suction and washed with 200 cc. of water. The washed precipitate was then suspended in the water and dissolved by the addition of alkali to a slight basic reaction to litmus. The solution turned orange red in color. The protein was reprecipitated with hydrochloric acid and again dissolved in the minimal quantity of alkali. The nitrogen content was 3.08 mgs. per cc.

B. Preparation of diazoparamino-benzoic acid proteins.

(1) The gelatin compounds.

Ten grams of gelatin were dissolved in 100 cc. of hot water. The solution was gelled in the ice box, diced, and mixed with 200 cc. of cold N/1 sodium carbonate. Paraaminobenzoic acid, 1.37 gms. (5 times the theoretical 0.002.) was diazotized with N/2 sodium nitrate about (20 cc.) at 0-3 C, starch iodide paper was used as indicator. The cooled diazo compound was then added to the protein-carbonate mixture and allowed to couple over night in the ice box.

The cubes were then filtered off on cheese cloth, with cold distilled water, melted and the protein compound precipitated by the addition of 1 liter of acidified alcohol. The precipitate was then filtered and redissolved as before. The process was repeated twice. The protein solution was then filtered, subsequently sterilized by Berkefeld filtration and then made isotonic with sodium chloride.

(2) The preparation of diazoparaminobenzoic acid horse serum.

This preparation was made according to the directions of Landsteiner and Van der Scheer[12]: Paraaminobenzoic acid, 1.77 gms, was dissolved in 10 cc of water and 5 cc of 7N HCl and diazotised as usual. The solution was diluted with ice water to a volume of 100 cc. and poured into 100 cc of horse serum to which 50cc. of normal sodium hydroxide had been added. Coupling was allowed to take place for ten minutes at about

0° C. The proteins were then precipitated with hydrochloric acid centrifuged down and suspended in a small quantity of water. On addition of normal sodium hydroxid and stirring the solution becomes very viscous and jelly-like. A large quantity of alcohol was added and subsequently enough hydrochloric acid to flocculate the protein.

The precipitate was filtered and treated twice again in the same manner. In order to remove the alcohol, the proteins were again brought into solution with alkali and, after dilution, reprecipitated with hydrochloric acid and centrifuged. The precipitate was then dissolved in water at slight alhaline reaction, made up to a volume of 190 cc., and sterilized by Berkfeld filtration and made isotonic with sodium chloride.

(C) Preparation of anilin-proteins.

(1) The gelatin compound.

In this preparation a simpler procedure was used. The writer is indebted to Dr. Matthew A. Derow for this procedure. Anilin, 0.93 grams, dissolved in 10 cc. of water with 2.5 cc of concentrated hydrochloric acid, was diazotized as previously described and coupled with gelatin. Instead of repeated precipitation the cubes are dialysed directly in a cheese cloth bag. The cubes act as their own membrane in dialysis and do not dissolve in cold tap water. After dialysis over night the cubes swelled ot twice their original size. The cubes were then washed several times with distilled water and then melted, made up to a volume of 200 cc. and made isotonic with NaCl. Since gelatin solutions are very hard to filter through a Berkefeld, sterilizaton was accomplished by heating the gelatin for 45 minutes at 95° C. The sterility of the protein was controlled by streaking agar slants and incubating at 20° and 37°.

(2) Preparation of anilin-horse-serum.

This preparation was made in the same way as the para-aminobenzoic acid compound except that 100 cc. of normal sodium carbonate was used instead alkali. Equivalent quantities were used throughout.

(c) Preparation of gallic-pyridine.

(1) The gelatin solution.

In this preparation a similar procedure was used. The water is treated with 0.5 gm. gelatine m. Dorse for 1½ preparation, 0.05 gram, dissolved in 10 cc. of water with 0.5 cc of concentrated hydrochloric acid, was discarded as previously described and coupled with gelatin. Instead of repeated precipitation the mass was dialyzed directly in a dialyzer for 3 hrs. The tubes are in their own membrane in 500 cc and 50 cc distilled in cold the water. After dialyzing over night the tubes emptied of their short or final size. The solution were then washed several times with distilled water and then mixed, made up to a volume of 500 cc. and made isotonic with NaCl. These gelatin solutions are very hard to filter through a Berkefeld, sterilization is accomplished by heating the solution for 45 minutes at 95°. The sterilizing of the protein is controlled by giving up after minute and hours. 1½ at 50° and 37°.

(2) Preparation of gallic-pyridine.

This preparation was made in the same manner as the parent substance, using one and about 1% 100 cc. of normal sodium acetate was used instead water. Various quantities were used throughout.

(D) Immunization of rabbits.

The following is a record of the injection schedule used for the nitrogelatin.

Date	No. of rabbit	Volume injected	Route
3/9/34	680,703,704	1.5 cc	Intravenous
3/13/34	" " "	2 cc	"
3/14/34	" " "	2 cc	"
3/20/34	680,704	3 cc	"
	703	3 cc	Intraabdominal
3/21/34	680,704	3 cc	"
	703	3 cc	"
3/22/34	680,704	3 cc	"
	703	3 cc	"
3/27/34	680,703,704	5 cc	"
4/1/34	680,703,704	5 cc	"
4/3/34	" " "	5 cc	"
4/10/34	Final bleeding		

The blood serum was tested for precipitins after 9 injections, using nitrogelatin, nitrocasein and nitroalbumin. Dilutions of the antigens were made from 10-100,000. No precipitins were demonstrated.

Three rabbits were injected with paraminobenzoic acid gelatin over a period of approximately four months with total of 30 doses. The following is a record of the injection schedule.

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The following is a record of the injection schedule used for the nitrogenation.

Date	No. of rabbit	Volume injected	Route
3/9/24	680,705,704	1.5 cc	Intravenous
3/13/24	" " "	2 cc	"
3/14/24	" " "	2 cc	"
3/20/24	680,704	3 cc	"
	703	3 cc	Intraabdominal
3/21/24	680,704	3 cc	"
	703	3 cc	"
3/28/24	680,704	3 cc	"
	703	3 cc	"
3/27/24	680,703,704	5 cc	"
4/1/24	680,703,704	5 cc	"
4/8/24	" " "	5 cc	"
4/10/24	Final bleeding		

The blood serum was tested for precipitins after a injection, using nitrogenated, nitrosated and nitronitrosated. Dilution of the antisera were as a from 10-100,000. No precipitins were demonstrated.

Three rabbits were injected with paranitrobenzoic acid gelatin over a period of approximately four months with total of 30 doses. The following is a record of the injection schedule.

Date	No. of rabbit	Volume injected	Route
10/29/34	760.761,762	5 cc	Intravenous
10/30/34	" " "	"	"
11/8/34	" " "	"	"
11/9/34	" " "	"	"
11/10/34	" " "	"	"
11/16/34	" " "	1"cc	"
11/17/34	" " "	"	"
11/30/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/1/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/7/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/8/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/20/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/21/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/27/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
" " "	Trial bleeding		
12/28/34	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
12/2/34	760,761	1 cc	Intravenous

Date	No. of rabbit	Volume injected	Route
10/29/24	760,762	5 cc	Intravenous
10/30/24	" " "	"	"
11/6/24	" " "	"	"
11/9/24	" " "	"	"
11/13/24	" " "	"	"
11/25/24	" " "	1 cc	"
11/17/24	" " "	"	"
11/20/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/1/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/7/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/9/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/20/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/21/24	760,761	cc	Intravenous
" " "	762	2 cc	Intrasubdominal
12/27/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal
" " "	Final bleeding		
12/28/24	760,761	1 cc	Intravenous
" " "	762	2 cc	Intrasubdominal

Date	No. of rabbit	Volume injected	Route
1/3/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/4/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/11/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/12/35	760,761	1 cc	Intravenous
" " "	762	2 cc	Intraabdominal
1/17/35	760,761,762	2 cc	Intraabdominal
1/18/35	" " "	" "	"
1/19/35	" " "	" "	"
	1 week rest period		
2/1/35	760,761,762	1 cc	Intraabdominal
2/2/35	" " "	" "	"
2/7/35	" " "	½ cc	Intravenous
2/8/35	" " "	" "	"
2/9/35	" " "	" "	"
2/14/35	" " "	" "	"
2/15/35	" " "	" "	"
2/16/35	" " "	" "	"
2/22/35	Final bleeding		

After the fourteenth injection a trial bleeding was made and precipitin tests tried. Antigen dilution from 10 to 100,000 were made using gelatin paraminobenzoic acid and the horseserum compound. No intrafacial rings were observed after fifteen minutes or one hour at room temperature. The tubes were mixed

Date	No. of rabbit	Volume injected	Route
1/3/35	750,751	1 cc	Intravenous
" "	752	2 cc	Intracerebral
1/4/35	750,751	1 cc	Intravenous
" "	752	2 cc	Intracerebral
1/11/35	750,751	1 cc	Intravenous
" "	752	2 cc	Intracerebral
1/12/35	750,751	1 cc	Intravenous
" "	752	2 cc	Intracerebral
1/17/35	750,751,752	2 cc	Intracerebral
1/18/35	" " "	" "	"
1/19/35	" " "	" "	"

I week rest period

2/ /35	750,751,752	1 cc	Intracerebral
2/2/35	" " "	"	"
2/7/35	" " "	½ cc	Intravenous
2/8/35	" " "	" "	"
2/9/35	" " "	"	"
2/14/35	" "	" "	"
2/15/35	" " "	" "	"
2/16/35	" " "	" "	"
2/22/35	Final bleeding		

After the fourteenth injection a final bleeding was made and precipitin tests made. Antisera titers from 1:1 to 1:10,000 were made using rabbits parathyphoid and the homologous compound. No interstitial rings were observed after fifteen minutes or one hour at room temperature. The tubes were

and allowed to stand over night in the ice box. No precipitins could be found the next morning. The following morning precipitates were observed.

Serum		Dilutions of horse serum paraaminobenzoic acid				
		10	100	1000	10.000	100,000
761	Immune	+++	++	-	-	-
	Normal	++	++	-	-	-
762	Immune	+++	+++	±	-	-
	Normal	±	++	+	±	-

The plus signs indicate relative amounts of precipitate.

It was thought desirable to repeat these reactions. The tests were repeated using horse serum as an additional antigen. An attempt was made to duplicate the previous conditions in so far as possible. The results were completely negative. A subsequent precipitin test made after the final bleeding was also completely negative.

Three rabbits were also injected with analingelatin. The following is a record of the injection schedule.

Date	Number of rabbit	Amount	Route
12/20/34	763,766,767	½ cc	Intravenous
12/21/34	" " "	"	"
12/27/34	" " "	1 cc	"
12/28/34	" " "	"	"
1/3/35	" " "	"	"
1/4/35	" " "	"	"

and allowed to stand over night in the ice box. The precipitins could be found the next morning. The following reactions were observed.

Serum	Dilutions of horse serum pneumococcus soluble				
	10	100	1000	10 000	100,000
Vol. Immune	+++	++	-	-	-
Normal	++	++	-	-	-
Vol. Immune	+++	+++	±	-	-
Normal	±	++	+	±	-

The plus signs indicate relative amounts of precipitate.

It was thought desirable to repeat these reactions. The tests were repeated using her o serum as an additional antigen. An attempt was made to duplicate the previous conditions in so far as possible. The results were completely negative. A subsequent precipitin test made after the final bleeding was also completely negative.

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Date	Number of rabbit	Amount	Route
12/20/34	765,766,767	½ cc	Intravenous
12/21/34	" " "	"	"
12/27/34	" " "	1 cc	"
12/28/34	" " "	"	"
1/3/35	" " "	"	"
1/4/35	" " "	"	"

Date	Number of rabbit	Amount	Route
1/11/35	763,766,767	1 cc	Intravenous
1/12/35	" " "	"	"
1/17/35	" " "	"	"
1/18/35	" " "	"	"
1/19/35	" " "	"	"
1/24/35	" " "	"	"
1/25/35	" " "	"	"
2/1/35	" " "	2 cc	Intraabdominal
2/2/35	" " "	"	"
2/7/35	" " "	"	"
2/8/35	" " "	"	"
2/9/35	" " "	"	"
2/14/35	" " "	"	"
2/15/35	" " "	"	"
2/16/35	" " "	"	"
2/23/35	Final bleeding		

Precipitin tests were tried using horse serum anilin and the corresponding gelatin compound in dilutions of 10 to 100,000. The results were completely negative.

Date	Number of rabbit	Amount	Route
1/11/35	765,766,767	1 cc	Intravenous
1/12/35	" " "	"	"
1/13/35	" " "	"	"
1/14/35	" " "	"	"
1/19/35	" " "	"	"
1/20/35	" " "	"	"
1/24/35	" " "	"	"
2/1/35	" " "	2 cc	Intraperitoneal
2/2/35	" " "	"	"
2/7/35	" " "	"	"
2/8/35	" " "	"	"
2/9/35	" " "	"	"
2/14/35	" " "	"	"
2/15/35	" "	"	
2/16/35	" " "	"	"
2/22/35	Final bleeding		

Precipitin tests were tried until both before antisera and the pulmonary extract were coagulated. The results were completely negative.

III. Summary.

Gelatin is non-antigenic. Some compounds have been prepared in which gelatin is claimed to be antigenic. Other investigators report only combining reactions with synthetic gelatin compounds. Aromatic amino acids are supposed to be necessary for antigenicity. Additional considerations about collagen introduce the possibility of a new concept. Analin, paraaminobenzoic acid and nitro gelatins have been prepared and injected. The results so far have been absolutely negative.

III. Summary.

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